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Detecting Potentially Virulent *Vibrio vulnificus* Strains in Raw Oysters by Quantitative Loop-Mediated Isothermal Amplification▽

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Vibrio vulnificus **is a leading cause of seafood-related deaths in the United States. Sequence variations in the virulence-correlated gene (*vcg*) have been used to distinguish between clinical and environmental *V. vulnificus* strains, with a strong association between clinical ones and the C sequence variant (*vcgC*). In this study, *vcgC* was selected as the target to design a loop-mediated isothermal amplification (LAMP) assay for the rapid, sensitive, specific, and quantitative detection of potentially virulent *V. vulnificus* strains in raw oysters. No false-positive or false-negative results were generated among the 125 bacterial strains used to evaluate assay specificity. The detection limit was 5.4 CFU per reaction for a virulent *V. vulnificus* strain (ATCC 33815) in pure culture, 100-fold more sensitive than that of PCR. In spiked raw oysters, the assay was capable of detecting 2.5×10^3 CFU/g of *V. vulnificus* ATCC 33815, while showing negative results for a nonvirulent *V. vulnificus* strain (515-4c2) spiked at 10^7 CFU/g. After 6 h of enrichment, the LAMP assay could detect 1 CFU/g of the virulent *V. vulnificus* strain ATCC 33815. Standard curves generated in pure culture and spiked oysters suggested a good linear relationship between cell numbers of the virulent *V. vulnificus* strain and turbidity signals. In conclusion, the LAMP assay developed in this study could quantitatively detect potentially virulent *V. vulnificus* in raw oysters with high speed, specificity, and sensitivity, which may facilitate better control of *V. vulnificus* risks associated with raw oyster consumption.**

Vibrio vulnificus is a Gram-negative, halophilic bacterium that inhabits warm coastal and estuarine waters worldwide and occurs in high numbers in filter-feeding bivalve mollusks such as oysters and clams (29). This bacterium is an uncommon but serious cause of human illness due to the consumption of raw or undercooked seafood, especially oysters (5). Following ingestion of raw oysters or exposure of open wounds to seawater, *V. vulnificus* infection may rapidly develop (<24 h) into primary septicemia and wound infection, two fatal diseases with mortality rates of over 50% and 25%, respectively (4, 29). As a matter of fact, *V. vulnificus* has been regarded as the predominant cause (95%) of seafood-related deaths in the United States, responsible for approximately 30 deaths annually (5, 29). Additionally, the CDC's recent FoodNet report suggested a continued increase in *Vibrio* incidences since 2001, pointing to a need for improved prevention measures (5). In this regard, rapid and reliable detection methods are particularly needed to facilitate better control of potential *V. vulnificus* risks in raw oysters.

As an opportunistic human pathogen, *V. vulnificus* causes fatal infections predominantly among at-risk consumers, such as persons with immunocompromising conditions, diabetes, and an elevated serum iron concentration due to chronic liver disease or alcohol abuse (39). Besides host susceptibility, epidemiological data also support that only a small percentage of *V. vulnificus* strains in oysters are virulent (6, 21, 22, 41). Therefore, it is desirable that detection methods selectively target virulent *V. vulnificus* strains for accurate risk assessment and control. However, numerous *V. vulnificus* virulence factors examined to date appear to be present in both clinical and environmental strains (12, 22), and currently, there is a lack of unique virulence biomarkers that can be used to screen for virulent *V. vulnificus* strains in raw oysters.

Through bacterial genotyping studies, polymorphisms in several *V. vulnificus* loci have been used to group the *V. vulnificus* population into two genotypes, clinical (i.e., virulent) and environmental (i.e., nonvirulent). These loci include the virulence-correlated gene *vcg* (36, 43), 16S rRNA (2, 27), the siderophore-encoding *viuB* gene (31), the cytolysin-hemolysin gene *vvhA* (38), the capsular polysaccharide operon (7), and the pilus type IV assembly gene *pilF* (34). Using *vcg*, among 55 randomly selected *V. vulnificus* strains, 90% of strains with the *vcgC* sequence variant were clinical isolates, and 93% of environmental isolates had the *vcgE* sequence variant (36). Recently, by aligning eight loci using multiple genome-sequenced *V. vulnificus* strains, the *vcgC-vcgE* dimorphism was found in all eight loci, including housekeeping genes (35). Therefore, *vcgC* may serve as a reliable biomarker to screen for potentially virulent *V. vulnificus* strains.

In most studies using biomarkers to differentiate and detect potentially virulent *V. vulnificus* strains, PCR or real-time quantitative PCR (qPCR) assays were used (3, 7, 14, 40, 42). However, use of both PCR and qPCR requires an expensive thermal cycler, which hinders the wide application of such assays. Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification assay that utilizes four to six primers to specifically recognize six to eight regions of the target DNA sequence and amplifies millions of DNA copies under isothermal conditions (60 to 65°C) within an hour (28). Since it is isothermal, no expensive thermal cycling instrument is needed. Additionally, by monitoring the turbidity change

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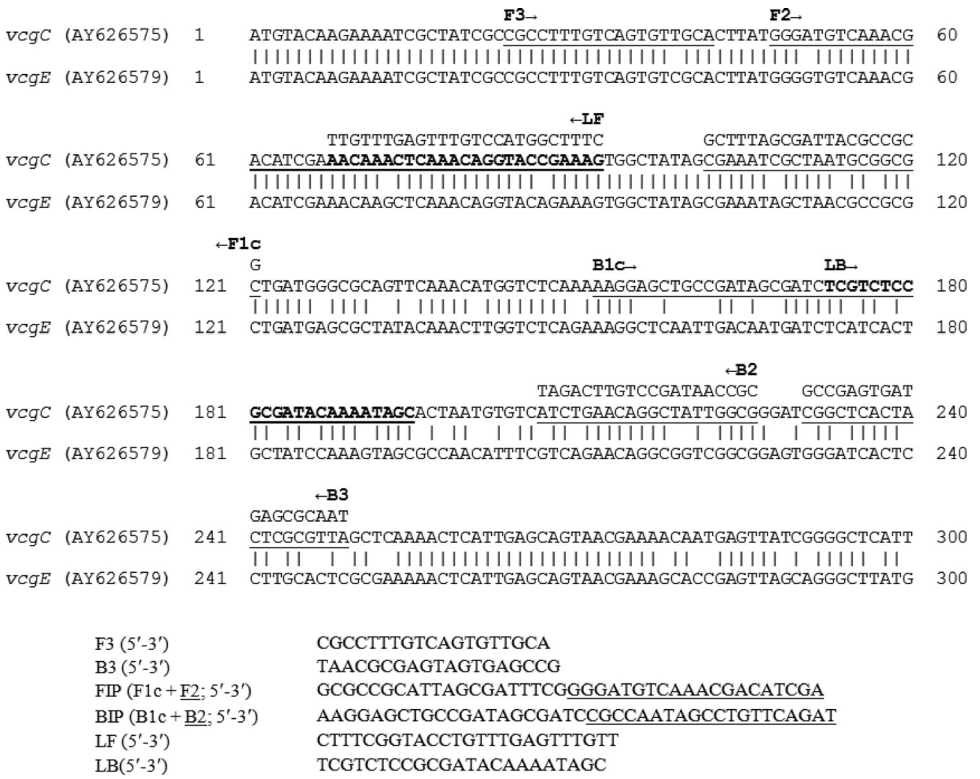

FIG. 1. Partial nucleotide sequence alignment of the *Vibrio vulnificus* virulence-correlated gene (*vcg*) types C and E (GenBank accession numbers AY626575 and AY626579, respectively). F3 and B3 are the forward and backward outer primers, respectively. FIP and BIP are the forward and backward inner primers, respectively. LF and LB (underlined and in boldface) are the forward and backward loop primers, respectively. F1c and B1c are the complementary sequences of F1 and B1, respectively.

due to the formation of a by-product (magnesium pyrophosphate), LAMP can be quantitative, as turbidity signals correlate with the number of DNA copies in the sample (24, 25). Recently, LAMP has been applied in the detection of multiple bacterial and viral agents of food safety concern, such as *Campylobacter*, *Salmonella*, pathogenic *Escherichia coli*, *Vibrio*, and norovirus (8, 13, 15, 18, 19, 44, 46). Very recently, a LAMP assay targeting the *V. vulnificus vcgC* gene was developed for the detection of virulent *V. vulnificus* from coastal seawater and was found to be sensitive and accurate (23). However, the quantitative capability of LAMP and the application of the assay in raw oysters were not examined.

The objective of this study was to develop and evaluate a *vcgC*-based LAMP assay suitable for the quantitative detection of potentially virulent *V. vulnificus* strains in raw oysters, which will serve as a rapid and reliable tool to facilitate better control of *V. vulnificus* risks associated with raw oyster consumption.

MATERIALS AND METHODS

Bacterial strains and DNA template preparation. *V. vulnificus* clinical strain ATCC 33815 (*vcgC* genotype) was used for assay optimization and sensitivity testing. *V. vulnificus* environmental strain 515-4c2 (*vcgE* genotype), originally isolated from oysters, was used to verify assay specificity in spiked oysters. Additionally, 83 *V. vulnificus* strains (33 *vcgC*-type and 50 *vcgE*-type strains), 30 other *Vibrio* spp., and 12 non-*Vibrio* strains as described in our previous reports (15, 16) were used to evaluate assay specificity. The *vcgC* and *vcgE* genotypes of the *V. vulnificus* strains were determined using PCR as described previously (36). *Vibrio* strains were cultured at 35°C overnight on Trypticase soy agar (TSA) or in Trypticase soy broth (TSB) (BD Diagnostic Systems, Sparks, MD) supplemented with 2% NaCl. Non-*Vibrio* strains were grown on TSA or blood agar (BD Diagnostic Systems), and *Campylobacter* strains were grown under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2).

For specificity testing, DNA templates were prepared by suspending several single colonies grown on appropriate agar plates in 500 μl of TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA; Sigma-Aldrich, St. Louis, MO) and heating the suspension at 95°C for 10 min in a dry heating block. After centrifugation at 12,000 × *g* for 2 min, the supernatants were stored at −20°C until use. To prepare templates for sensitivity testing, an overnight *V. vulnificus* ATCC 33815 culture was diluted 50-fold in fresh TSB and incubated at 35°C for 5 h with shaking at 100 rpm to reach mid-log phase. Ten-fold serial dilutions in phosphate-buffered saline (PBS; Sigma-Aldrich) were made, and aliquots (500 μl) of each dilution were used to prepare DNA templates similarly by heating them at 95°C for 10 min. The exact number of cells was determined by standard plate counting.

Target and LAMP primer design. Sequences of *V. vulnificus vcgC* and *vcgE* were obtained from the GenBank database (accession numbers AY626575 and AY626579, respectively) and aligned using BLAST (http://www.ncbi.nih.gov/blast). A set of six primers, two outer (F3 and B3), two inner (forward inner primer [FIP] and backward inner primer [BIP]), and two loop (LF and LB), that recognize eight distinct regions of the *vcgC* sequence (Fig. 1) were designed using the Primer Explorer software (version 4; Fujitsu Limited, Japan [http://primerexplorer.jp/e]). FIP consisted of the complementary sequence of F1 and the sense sequence of F2, whereas BIP was a combination of the complementary sequence of B1 and the sense sequence of B2. LF and LB were two loop primers designed to accelerate the LAMP reaction. Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

LAMP reaction and optimization. By following recommendations of the manufacturer (Eiken Chemical Co., Ltd., Tokyo, Japan), the LAMP prototypic reaction mix in a total volume of 25 μl contained 1× ThermoPol reaction buffer (New England Biolabs, Ipswich, MA), 6 mM $MgSO_4$, 0.8 M betaine (Sigma-Aldrich), 1.4 mM each deoxynucleotide triphosphate (dNTP), 0.2 μM F3 and B3, 1.6 μM FIP and BIP, 0.8 μM LF and LB, 8 U of Bst DNA polymerase (New

England Biolabs), and 2 μl DNA template. One positive control and one negative control were included in each LAMP run. The LAMP reaction was carried out at 63°C for 1 h and terminated at 80°C for 5 min in a real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan) which acquired turbidity readings at 650 nm every 6 s. The time threshold (T_T) values (in minutes) were determined when the turbidity increase measurements (the differential value of the moving average of turbidity) exceeded a threshold of 0.1.

LAMP optimization was performed using *V. vulnificus* ATCC 33815 by varying each assay parameter one at a time, which included the concentrations of $MgSO_4$ (2, 4, 6, 8, and 10 mM), betaine (0, 0.2, 0.4, 0.6, 0.8, and 1 M), dNTP (0.4, 0.8, 1.2, 1.6, and 2 mM), enzyme (2, 4, 6, 8, and 10 U), outer primers (0.05, 0.1, 0.2, 0.3, and 0.4 μM), inner primers (1.2, 1.4, 1.6, 1.8, and 2 μM), and loop primers (0.2, 0.4, 0.6, 0.8, and 1 μM) and the assay temperatures (60, 63, and 65°C). Each optimization experiment was repeated three times.

PCR and qPCR. As a comparison, two PCR assays targeting the *V. vulnificus vcgC* gene were carried out, one using the LAMP outer primers (F3/B3) designed in this study and the other one using a set of primers (P1/P3) described previously (36). The PCR mix (25 μl total) consisted of 1× PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.5 U of GoTaq Hot Start polymerase (Promega, Madison, WI), 0.2 μM each primer, and 2 μl of DNA template. The PCR was conducted by using 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min in a Bio-Rad C1000 thermal cycler (Hercules, CA). The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide, visualized under UV light, and documented with a Gel Doc XR system (Bio-Rad).

Additionally, a SYBR green I-based qPCR assay using LAMP outer primers F3/B3 was also conducted. The qPCR reagent mix (25 μl total) contained 1× FastStart SYBR green master mix (Roche Applied Science, Indianapolis, IN), 0.2 μM each primer, and 2 μl of DNA template. The qPCRs were carried out using 45 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 25 s in a SmartCycler II system (Cepheid, Sunnyvale, CA). Fluorescence readings were obtained using the 6-carboxyfluorescein (FAM) channel (excitation at 450 to 495 nm and detection at 510 to 527 nm), followed by melting curve analysis from 60°C to 94°C at increments of 0.2°C per second. The cycle threshold (C_T) values were obtained when the fluorescence readings crossed a threshold of 30 units.

LAMP specificity and sensitivity. A total of 125 bacterial strains described above were used to determine LAMP specificity. Aliquots (2 μl) of each DNA template were subjected to both LAMP and PCR amplifications. Specificity tests were repeated twice.

Additionally, to confirm the specific amplification of *V. vulnificus vcgC*, LAMP products were digested with 10 U of RsaI (New England Biolabs). Digested and undigested LAMP products were analyzed side by side using electrophoresis on a 2% agarose gel containing ethidium bromide and visualized under UV light.

To determine LAMP sensitivity, aliquots (2 μl) of the 10-fold serially diluted *V. vulnificus* ATCC 33815 templates prepared as described above were subjected to both LAMP and PCR/qPCR amplifications. Sensitivity tests were repeated five times.

Quantification of virulent *V. vulnificus* cells in spiked oysters. Oyster samples were obtained from a local seafood processing company during November to December 2009 and analyzed within 24 h. Twelve oysters (ca. 300 g) were homogenized in a food stomacher (model 400; Tekmar Company, Cincinnati, OH) for 2 min, and each 25-g portion of the homogenate (one sample) was mixed with 225 ml of alkaline peptone water (APW; BD Diagnostic Systems) to produce a 1:10 oyster-APW homogenate ratio. The homogenate was analyzed for the presence/absence of natural *V. vulnificus* by culture by following the methods described previously (17).

Confirmed *V. vulnificus*-negative oyster homogenates were inoculated with 10-fold serially diluted mid-log-phase *V. vulnificus* ATCC 33815 cells prepared as described above and analyzed immediately. Briefly, 1 ml of *V. vulnificus* ATCC 33815 cell suspension was added to 250 ml of 1:10 oyster-APW homogenate and mixed thoroughly. An aliquot (1 ml) of the spiked oyster homogenate was centrifuged at 900 × *g* for 1 min to remove oyster tissues, followed by another centrifugation at 10,000 × *g* for 5 min to pellet bacterial cells. The pellets were suspended in 100 μl of TE buffer and boiled for templates as described above. Aliquots (2 μl) were used for both LAMP and PCR/qPCR amplifications. In addition to direct testing, enrichment was also performed by incubating the spiked oyster homogenate at 35°C for 6 h. After enrichment, the homogenate was processed similarly to the process described above for direct testing. Three sets of independent oyster spiking experiments were performed, and the LAMP, PCR, and qPCR assays were repeated three times for each set of inoculations.

In addition, spiking with a nonvirulent (*vcgE* genotype) *V. vulnificus* strain (515-4c2) at 10^7 CFU/g was conducted and tested similarly to verify that LAMP could specifically detect only potentially virulent (*vcgC* genotype) *V. vulnificus* strains.

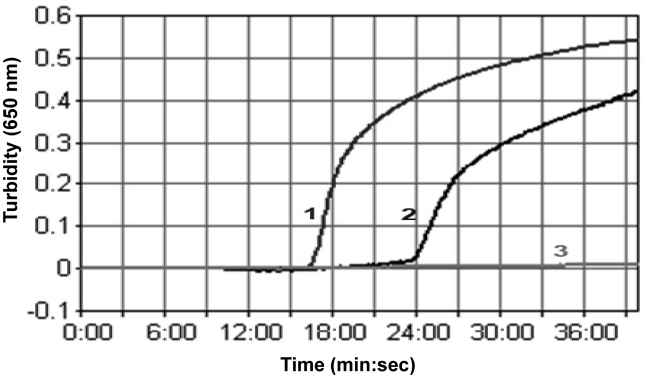

FIG. 2. Comparison of LAMP turbidity graphs obtained when running the assay under optimized or prototypic conditions, using *Vibrio vulnificus* ATCC 33815 as the template. Samples 1 and 2 were run under optimized and prototypic conditions, respectively. Sample 3 is water.

Data analysis. For specificity data, means and standard deviations of T_T values for LAMP were calculated by using Microsoft Excel software (Microsoft, Seattle, WA). For sensitivity data, means and standard deviations of T_T or C_T values for qPCR when detecting 10-fold serially diluted *V. vulnificus* ATCC 33815 in pure culture and spiked oyster homogenates were calculated similarly by using Microsoft Excel. The detection limits (number of CFU/reaction in pure culture or number of CFU/g in spiked oysters) were presented as the lowest numbers of cells that could be detected by the assays. In spiked oyster homogenates, the number of CFU/reaction was calculated by using the following formula: (number of CFU per g × 25 g)/(250 × 10 × 2 × 10^{-3}), i.e., number of CFU per g × 2 × 10^{-3}.

Standard curves to quantify *V. vulnificus* cells in pure culture and spiked oyster homogenates were generated by plotting T_T or C_T values against the number of log CFU/reaction for pure culture or the number of log CFU/g for spiked oysters, and linear regression was calculated by using Microsoft Excel. Quantitative capabilities of the assays were derived based on the correlation coefficient (R^2) values derived from the standard curves.

RESULTS

Optimized *vcgC*-LAMP assay. The optimized LAMP reaction mix deviated from the prototypic one in terms of the following parameters: betaine (0 versus 0.8 M in the prototype), dNTP (1.2 mM versus 1.4 mM in the prototype), F3 and B3 (0.05 μM versus 0.2 μM in the prototype), FIP and BIP (2 μM versus 1.6 μM in the prototype), LF and LB (1 μM versus 0.8 μM in the prototype), and Bst DNA polymerase (10 U versus 8 U in the prototype). Additionally, LAMP reactions carried out at 65°C for 40 min were found to be optimal, in contrast to reactions being optimal at 63°C for 60 min in the prototype.

Figure 2 shows amplification graphs generated using the optimized (line 1) and prototypic (line 2) conditions for *V. vulnificus* ATCC 33815. Besides decreasing T_T values (18.3 min under the optimized condition versus 26.3 min under the prototypic one), the turbidity signal intensity was also markedly stronger when the optimized condition was used.

LAMP specificity. Among 125 bacterial strains used to evaluate LAMP specificity, no false-positive or false-negative results were observed. The T_T values for the 33 *vcgC*-type *V. vulnificus* strains ranged from 16.1 to 22.3 min, with an average

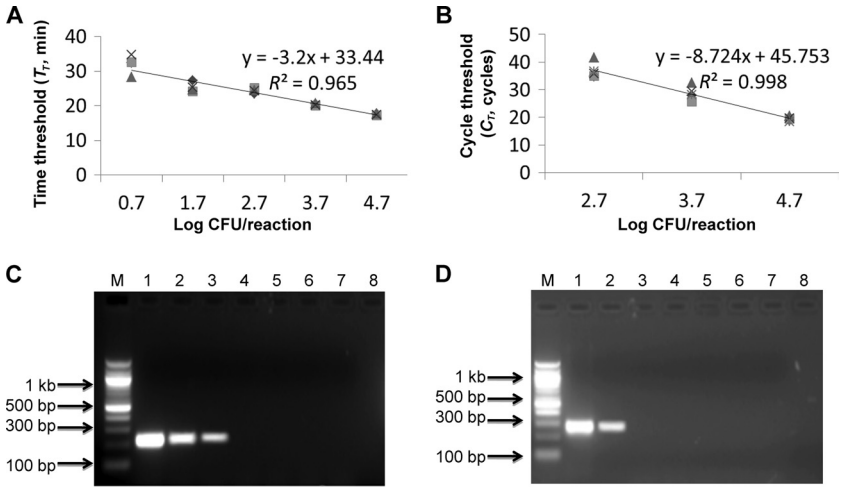

FIG. 3. Comparison of sensitivities and quantitative capabilities of the LAMP, qPCR, and PCR assays (sensitivities only for the PCR assay) when testing the 10-fold serially diluted virulent *Vibrio vulnificus* strain ATCC 33815. (A) LAMP standard curve generated based on five independent repeats; (B) qPCR standard curve generated based on five independent repeats; (C) representative gel image generated by PCR using the F3/B3 primers (226-bp amplicons); (D) representative gel image generated by PCR using the P1/P3 primers (278-bp amplicons). (C and D) Lanes 1 to 7 correspond to *V. vulnificus* ATCC 33815 cells ranging from 5.4×10^4 to 5.4×10^{-2} CFU/reaction, lane 8 is water, and lane M is a 100-bp molecular mass marker (New England Biolabs).

of 18.2 ± 1.5 min. For the other 92 strains consisting of 50 *vcgE*-type *V. vulnificus*, 30 other *Vibrio* spp., and 12 non-*Vibrio*, no T_T values were obtained, indicating negative results for the *vcgC*-LAMP assay. Similarly, PCR assays included as a comparison successfully detected 33 *vcgC*-type *V. vulnificus* strains while showing negative results for the other 92 strains (data not shown).

In addition, RsaI digestion of the LAMP products yielded the expected fragments of 167 bp and 270 bp (data not shown), suggesting specific amplification of the target *vcgC* sequence by LAMP.

LAMP sensitivity and quantitative capability. Figure 3 shows standard curves generated when testing the 10-fold serially diluted virulent (*vcgC* genotype) *V. vulnificus* strain ATCC 33815 by *vcgC*-based LAMP (Fig. 3A) and qPCR (Fig. 3B), as well as PCR gels using F3/B3 (Fig. 3C) and the P1/P3 primers (Fig. 3D). For templates ranging from 5.4×10^4 to 5.4 CFU/reaction, the average T_T values based on five repeats fell between 17.5 and 31 min, with a detection limit of 5.4 CFU/reaction. In contrast, the *vcgC*-based qPCR assay had a detection limit of 5.4×10^2 CFU/reaction, with C_T values averaging between 19.4 and 36.8 cycles for templates ranging from 5.4×10^4 to 5.4×10^2 CFU/reaction, while melting temperatures consistently fell at around 80°C (data not shown). Similarly, the detection limits for the two PCR assays as shown on the gels (Fig. 3C and D) were 5.4×10^2 and 5.4×10^3 CFU/reaction for primer pairs F3/B3 and P1/P3, respectively. Therefore, LAMP was 100-fold more sensitive than PCR or qPCR.

Based on the standard curves for the *vcgC*-based LAMP and qPCR (Fig. 3A and B), correlation coefficients (R^2) of LAMP and qPCR were calculated to be 0.965 and 0.998, respectively, indicating excellent linear relationships between *vcgC*-type *V. vulnificus* cell numbers (numbers of log CFU/reaction) and the amplification signals (T_T or C_T values). PCR, on the other hand, is not quantitative.

Rapid and specific quantification of virulent *V. vulnificus* in spiked oysters. For oyster homogenates spiked with nonvirulent *V. vulnificus* strain 515-4c2, in three independent experiments, LAMP consistently gave negative results for samples spiked with an average cell concentration of 1.3×10^7 CFU/g, which was equivalent to 2.6×10^4 CFU in the reaction tube.

Figure 4 shows standard curves generated when testing the 10-fold serially diluted virulent (*vcgC* genotype) *V. vulnificus* strain ATCC 33815 in spiked oyster homogenates by using LAMP (Fig. 4A) and qPCR (Fig. 4B). In three independent

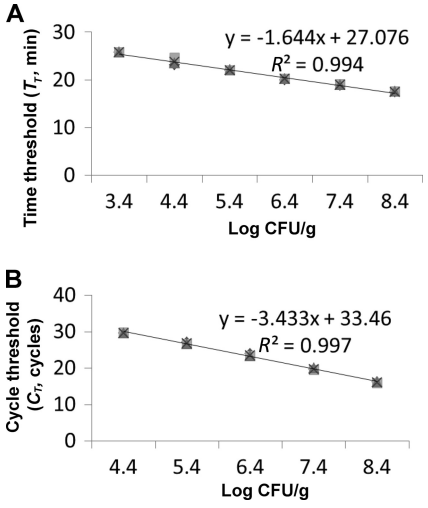

FIG. 4. Quantitative detection of virulent *Vibrio vulnificus* strain ATCC 33815 in spiked oysters by LAMP and qPCR. Three sets of independent spiking experiments were performed, and LAMP and qPCR assays were repeated three times for each set of inoculations. (A) LAMP standard curve based on three independent repeats; (B) qPCR standard curve based on three independent repeats.

spiking experiments, LAMP detected this virulent *V. vulnificus* strain at down to 2.5×10^3 CFU/g (equivalent to 5 CFU/reaction) in oyster homogenates, with average T_T values ranging from 17.5 to 25.7 min for samples spiked with between 2.5×10^8 and 2.5×10^3 CFU/g. In comparison, qPCR had a detection limit of 2.5×10^4 CFU/g (equivalent to 50 CFU/reaction), with average C_T values ranging from 16.1 to 29.7 cycles for samples spiked with between 2.5×10^8 and 2.5×10^4 CFU/g. For PCR, the detection limits were 2.5×10^6 and 2.5×10^7 CFU/g when using primer sets F3/B3 and P1/P3, respectively (data not shown), at least 100-fold less sensitive than those for LAMP or qPCR. The R^2 values ranged from 0.977 to 0.994 for LAMP and 0.995 to 0.997 for qPCR (Fig. 4), indicating excellent quantitative capability.

After 6 h of enrichment, the *vcgC*-based LAMP assay could consistently detect an initial spiking of 1 virulent *V. vulnificus* strain ATCC 33815 per gram of oyster homogenate, making it >1,000-fold more sensitive than direct testing without enrichment (data not shown). In contrast, the detection limits for both qPCR and the two PCR assays after 6 h of enrichment were at 1.2×10^2 CFU/g (data not shown).

DISCUSSION

LAMP technology has been applied previously to detect the total number of *V. vulnificus* strains by targeting species-specific genes such as *vvhA* (13, 15, 33) or *toxR* (26). Very recently, a LAMP assay targeting the *V. vulnificus* virulence-correlated gene type C (*vcgC*) was developed to detect potentially virulent *V. vulnificus* in coastal seawater; however, no loop primers were designed, the assay used was not quantitative, and the application in raw oysters was not examined (23). The *vcgC*-based LAMP assay developed in the present study is rapid (16 to 40 min), as accelerated by two loop primers, specific (no false-positive or false-negative results for 125 strains tested and no amplification from a nonvirulent *V. vulnificus* strain spiked at 10^7 CFU/g in oyster homogenates), sensitive (5.4 CFU/reaction in pure culture and 2.5×10^3 CFU/g in spike oysters), and quantitative ($R^2 = 0.965$ to 0.994). To our knowledge, this is the first report applying LAMP to detect and quantify potentially virulent *V. vulnificus* strains in raw oysters by targeting a virulence-associated biomarker.

Similar to two recently published LAMP and qPCR studies (3, 23), the *vcgC* gene was chosen in this study as the target to design primers for virulent *V. vulnificus* detection. Among all biomarkers used to date to differentiate the *V. vulnificus* population into clinical and environmental genotypes, *vcg* and 16S rRNA were the two used most extensively (2, 7, 11, 27, 35–37, 41, 43). In our recent study characterizing 349 *V. vulnificus* isolates from Louisiana Gulf and retail oysters using multiple biomarkers, *vcg* and 16S rRNA possessed the best overall agreement, approximately 90% (16). However, one problem associated with using 16S rRNA as the biomarker to differentiate *V. vulnificus* strains was the frequent observations of *V. vulnificus* strains possessing both clinical (type B) and environmental (type A) genotypes (7, 9, 11, 16, 37, 40, 41). On the other hand, the vast majority of studies of *vcg* reported mutual exclusivity of *vcgC* and *vcgE*, i.e., *V. vulnificus* strains possessed either *vcgC* or *vcgE* but not both (9, 16, 36, 37). Nonetheless, our repeated attempts to design LAMP assays based on 16S rRNA type B were not successful; both A- and B-type strains were amplified by LAMP, although 16S rRNA A-type strains needed more time (ca. 10 min) to achieve positive results (data not shown).

In the optimized LAMP reagent mix, betaine was completely eliminated, and concentrations of other reagents were slightly modified from those used in the prototype. Contrary to our findings, a few studies suggested that a higher betaine concentration resulted in elevated LAMP efficiency and increased target selectivity (28, 45). Betaine was capable of isostabilizing DNA and preventing secondary structure formation in the GC-rich region, thus reducing base stacking and promoting DNA amplification (20, 32). Therefore, the effect of betaine may be dependent upon the GC composition of each target sequence. Additionally, betaine has not been used as routinely in PCRs as in LAMP. A comparison of the two LAMP graphs (Fig. 2) in the present study clearly indicated that under optimized conditions, the LAMP reaction progressed faster, and the turbidity signals obtained were markedly stronger, which underscored the importance of performing LAMP optimization experiments for each primer set designed in order to achieve optimum LAMP efficiency.

The *vcgC*-LAMP assay developed in this study consistently detected down to 5.4 cells of a virulent *V. vulnificus* strain per reaction in pure culture. This level of sensitivity was 100-fold superior to those of the two PCR assays run in parallel. Previously published LAMP assays for total *V. vulnificus* detection had sensitivities of 1 to 20 CFU per test, 10- to 100-fold more sensitive than PCR (13, 15, 33). The recently published *vcgC*-based LAMP assay for virulent *V. vulnificus* detection reported a detection limit of 16 copies per reaction in pure culture, in contrast to a limit of 180 copies per reaction for PCR (23). This increased sensitivity of LAMP (by at least 10-fold) compared to that of PCR agreed with findings from many previous studies (8, 10, 18, 19). On the other hand, a comparison between LAMP and qPCR for *V. vulnificus* detection has not been made. When directly using LAMP outer primers as qPCR primers in a SYBR green I-based assay, qPCR was found to be 100-fold less sensitive than LAMP. Since no optimization was performed for this qPCR assay, the sensitivity could have been underestimated. Previously, qPCR was reported to detect 3 *V. vulnificus* cells using probe-based qPCR assays (30).

LAMP amplicons were commonly detected by gel electrophoresis, naked eye observation of turbidity or color change, and real-time turbidimeter monitoring, and among those, real-time turbidimeter monitoring is the only one that is potentially quantitative (15). However, very few studies have examined the quantitative capability of LAMP, including the recently developed *vcgC*-LAMP assay (23). One study monitoring ammonia-oxidizing bacteria using LAMP reported that it possessed good quantitative capability at between 10^4 and 10^{10} DNA copies (1). Two other studies demonstrated strong linear correlation coefficients ($R^2 = 0.94$ to 0.99) of LAMP in the detection of *Vibrio parahaemolyticus* and *V. vulnificus* in spiked oysters (8, 15). In the present study, the R^2 values were found to be 0.965 for virulent *V. vulnificus* cell concentrations between 10^4 and 10^0 CFU/reaction in pure culture and 0.977 to 0.994 for cells ranging from 10^8 to 10^3 CFU/g (10^5 to 10^0 CFU/reaction) in spiked oyster homogenates, suggesting excellent quantitative capabilities.

Without enrichment, the detection limit of the *vcgC*-LAMP assay for a virulent *V. vulnificus* strain in spiked oyster homogenates was 2.5×10^3 CFU/g (5 CFU/reaction), 10-fold more sensitive than that of qPCR and 10^3 to 10^4 more sensitive than those of the two PCR assays. In comparison, the recently reported *vcgC*-LAMP assay demonstrated a sensitivity of 20 copies of genomic DNA of a virulent *V. vulnificus* strain A1885 per reaction in seawater, whereas 300 copies were needed for PCR (23). After 6 h of enrichment, the LAMP developed in this study could consistently detect an initial spiking of 1 CFU/g of a virulent *V. vulnificus* strain, 100-fold more sensitive than either qPCR or PCR. This short-period enrichment procedure combined with simplified sample processing steps and rapid LAMP confirmation (<40 min) would make it possible to complete the analysis within an 8-h workday.

From a public health perspective, rapid and reliable determination of virulent *V. vulnificus* cells is much more desired in order to accurately assess the potential risks and implement timely controls. The *vcgC*-based LAMP assay developed in this study is a rapid, specific, sensitive, and cost-effective method for the detection and quantification of potentially virulent *V. vulnificus* strains in oysters. This assay may present a valuable tool for the oyster industry and regulatory agencies to better control the *V. vulnificus* risks associated with raw oyster consumption. Future testing with natural oyster samples is desired to further evaluate the performance of LAMP in a setting closer to application.

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REFERENCES

1. **Aoi, Y., M. Hosogai, and S. Tsuneda.** 2006. Real-time quantitative LAMP (loop-mediated isothermal amplification of DNA) as a simple method for monitoring ammonia-oxidizing bacteria. J. Biotechnol. **125:**484–491.
2. **Aznar, R., W. Ludwig, R. I. Amann, and K. H. Schleifer.** 1994. Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. Int. J. Syst. Bacteriol. **44:**330–337.
3. **Baker-Austin, C., et al.** 2010. Rapid *in situ* detection of virulent *Vibrio vulnificus* strains in raw oyster matrices using real-time PCR. Environ. Microbiol. Rep. **2:**76–80.
4. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine *Vibrio*. Clinical characteristics and epidemiology. N. Engl. J. Med. **300:**1–5.
5. **Centers for Disease Control and Prevention.** 2010. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food—10 states, 2009. MMWR Morb. Mortal. Wkly. Rep. **59:**418–422.
6. **Centers for Disease Control and Prevention.** 2010. Summary of human *Vibrio* cases reported to CDC, 2008. CDC, Atlanta, GA. http://www.cdc.gov/nationalsurveillance/PDFs/Jackson_Vibrio_CSTE2008_FINAL.pdf.
7. **Chatzidaki-Livanis, M., M. A. Hubbard, K. Gordon, V. J. Harwood, and A. C. Wright.** 2006. Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. Appl. Environ. Microbiol. **72:**6136–6141.
8. **Chen, S., and B. Ge.** 2010. Development of a *toxR*-based loop-mediated isothermal amplification assay for detecting *Vibrio parahaemolyticus*. BMC Microbiol. **10:**41.
9. **Drake, S. L., B. Whitney, J. F. Levine, A. DePaola, and L. A. Jaykus.** 2010. Correlation of mannitol fermentation with virulence-associated genotypic characteristics in *Vibrio vulnificus* isolates from oysters and water samples in the Gulf of Mexico. Foodborne Pathog. Dis. **7:**97–101.
10. **Fall, J., et al.** 2008. Establishment of loop-mediated isothermal amplification method (LAMP) for the detection of *Vibrio nigripulchritudo* in shrimp. FEMS Microbiol. Lett. **288:**171–177.
11. **Gordon, K. V., M. C. Vickery, A. DePaola, C. Staley, and V. J. Harwood.** 2008. Real-time PCR assays for quantification and differentiation of *Vibrio vulnificus* strains in oysters and water. Appl. Environ. Microbiol. **74:**1704–1709.
12. **Gulig, P. A., K. L. Bourdage, and A. M. Starks.** 2005. Molecular pathogenesis of *Vibrio vulnificus*. J. Microbiol. **43:**118–131.
13. **Han, F., and B. Ge.** 2008. Evaluation of a loop-mediated isothermal amplification assay for detecting *Vibrio vulnificus* in raw oysters. Foodborne Pathog. Dis. **5:**311–320.
14. **Han, F., and B. Ge.** 2010. Multiplex PCR assays for simultaneous detection and characterization of *Vibrio vulnificus* strains. Lett. Appl. Microbiol. **51:**234–240.
15. **Han, F., and B. Ge.** 2010. Quantitative detection of *Vibrio vulnificus* in raw oysters by real-time loop-mediated isothermal amplification. Int. J. Food Microbiol. **142:**60–66.
16. **Han, F., S. Pu, A. Hou, and B. Ge.** 2009. Characterization of clinical and environmental types of *Vibrio vulnificus* isolates from Louisiana oysters. Foodborne Pathog. Dis. **6:**1251–1258.
17. **Han, F., R. D. Walker, M. E. Janes, W. Prinyawiwatkul, and B. Ge.** 2007. Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. Appl. Environ. Microbiol. **73:**7096–7098.
18. **Hara-Kudo, Y., et al.** 2007. Sensitive and rapid detection of Vero toxin-producing *Escherichia coli* using loop-mediated isothermal amplification. J. Med. Microbiol. **56:**398–406.
19. **Hara-Kudo, Y., M. Yoshino, T. Kojima, and M. Ikedo.** 2005. Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. FEMS Microbiol. Lett. **253:**155–161.
20. **Henke, W., K. Herdel, K. Jung, D. Schnorr, and S. A. Loening.** 1997. Betaine improves the PCR amplification of GC-rich DNA sequences. Nucleic Acids Res. **25:**3957–3958.
21. **Jackson, J. K., R. L. Murphree, and M. L. Tamplin.** 1997. Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous populations in shellfish. J. Clin. Microbiol. **35:**2098–2101.
22. **Jones, M. K., and J. D. Oliver.** 2009. *Vibrio vulnificus*: disease and pathogenesis. Infect. Immun. **77:**1723–1733.
23. **Li, Y., et al.** 2010. A culture-free method for detection of *Vibrio vulnificus* from coastal seawater based on loop-mediated isothermal amplification targeting *vcgC* gene. Acta Oceanol. Sin. **29:**93–97.
24. **Mori, Y., M. Kitao, N. Tomita, and T. Notomi.** 2004. Real-time turbidimetry of LAMP reaction for quantifying template DNA. J. Biochem. Biophys. Methods **59:**145–157.
25. **Mori, Y., K. Nagamine, N. Tomita, and T. Notomi.** 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. **289:**150–154.
26. **Nemoto, J., et al.** 2008. Rapid detection of *Vibrio vulnificus* using a loop-mediated isothermal amplification method. Kansenshogaku Zasshi **82:**407–413.
27. **Nilsson, W. B., R. N. Paranjype, A. DePaola, and M. S. Strom.** 2003. Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. J. Clin. Microbiol. **41:**442–446.
28. **Notomi, T., et al.** 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. **28:**E63.
29. **Oliver, J. D.** 2006. *Vibrio vulnificus*, p. 349–366. *In* F. L. Thompson, B. Austin, and J. Swings (ed.), The biology of vibrios. ASM Press, Washington, DC.
30. **Panicker, G., and A. K. Bej.** 2005. Real-time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting *vvhA*. Appl. Environ. Microbiol. **71:**5702–5709.
31. **Panicker, G., M. C. Vickery, and A. K. Bej.** 2004. Multiplex PCR detection of clinical and environmental strains of *Vibrio vulnificus* in shellfish. Can. J. Microbiol. **50:**911–922.
32. **Rees, W. A., T. D. Yager, J. Korte, and P. H. von Hippel.** 1993. Betaine can eliminate the base pair composition dependence of DNA melting. Biochemistry **32:**137–144.
33. **Ren, C. H., C. Q. Hu, P. Luo, and Q. B. Wang.** 2009. Sensitive and rapid identification of *Vibrio vulnificus* by loop-mediated isothermal amplification. Microbiol. Res. **164:**514–521.
34. **Roig, F. J., E. Sanjuan, A. Llorens, and C. Amaro.** 2010. *pilF* polymorphism-based PCR to distinguish *Vibrio vulnificus* strains potentially dangerous to public health. Appl. Environ. Microbiol. **76:**1328–1333.
35. **Rosche, T. M., E. A. Binder, and J. D. Oliver.** 2010. *Vibrio vulnificus* genome suggests two distinct ecotypes. Environ. Microbiol. Rep. **2:**128–132.
36. **Rosche, T. M., Y. Yano, and J. D. Oliver.** 2005. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. Microbiol. Immunol. **49:**381–389.
37. **Sanjuan, E., B. Fouz, J. D. Oliver, and C. Amaro.** 2009. Evaluation of genotypic and phenotypic methods to distinguish clinical from environmental *Vibrio vulnificus* strains. Appl. Environ. Microbiol. **75:**1604–1613.
38. **Senoh, M., et al.** 2005. The cytotoxin-hemolysin genes of human and eel

pathogenic *Vibrio vulnificus* strains: comparison of nucleotide sequences and application to the genetic grouping. Microbiol. Immunol. **49:**513–519.
39. **Strom, M. S., and R. N. Paranjpye.** 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microbes Infect. **2:**177–188.
40. **Vickery, M. C., W. B. Nilsson, M. S. Strom, J. L. Nordstrom, and A. DePaola.** 2007. A real-time PCR assay for the rapid determination of 16S rRNA genotype in *Vibrio vulnificus*. J. Microbiol. Methods **68:**376–384.
41. **Warner, E., and J. D. Oliver.** 2008. Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. Appl. Environ. Microbiol. **74:**80–85.
42. **Warner, E. B., and J. D. Oliver.** 2008. Multiplex PCR assay for detection and simultaneous differentiation of genotypes of *Vibrio vulnificus* biotype 1. Foodborne Pathog. Dis. **5:**691–693.
43. **Warner, J. M., and J. D. Oliver.** 1999. Randomly amplified polymorphic DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other *Vibrio* species. Appl. Environ. Microbiol. **65:**1141–1144.
44. **Yamazaki, W., et al.** 2008. Development and evaluation of a loop-mediated isothermal amplification assay for rapid and simple detection of *Campylobacter jejuni* and *Campylobacter coli*. J. Med. Microbiol. **57:**444–451.
45. **Yeh, H. Y., C. A. Shoemaker, and P. H. Klesius.** 2005. Evaluation of a loop-mediated isothermal amplification method for rapid detection of channel catfish Ictalurus punctatus important bacterial pathogen *Edwardsiella ictaluri*. J. Microbiol. Methods **63:**36–44.
46. **Yoda, T., et al.** 2007. Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses. J. Med. Virol. **79:**326–334.